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Plasma levels of bone Gla-protein reflect bone formation in patients on chronic maintenance dialysis

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Plasma levels of bone Gla-protein reflect bone formation in patients on chronic maintenance dialysis. Predictive value of plasma levels of bone Gla-protein (BGP) for bone histology was evaluated in 30 chronically dialyzed patients. All patients underwent bone biopsies and serum biochemical parameters, including BGP, parathyroid hormone, and alkaline phosphatase; calcium and phosphate were measured at the time of biopsy. Bone histology showed renal osteodystrophy with low bone turnover and osteomalacia (LT-ROD) in 13 patients, and renal osteodystrophy with high bone turnover and prevailing hyperparathyroid bone disease (HT-ROD) in 17 patients. Values for BGP were above normal in LT-ROD (47.3 ± 7.9 vs. 6.8 ± 0.2 ng/ml) and extremely elevated in HT-ROD (831 ± 170 ng/ml). Similar differences were not found with the other serum biochemical parameters, even though BGP correlated with parathyroid hormone (r = 0.64) and alkaline phosphatase (r = 0.85). There were significant correlations between BGP and cellular and non-cellular parameters of bone formation (r = 0.73 to 0.91). Weaker or no correlations were found between BGP and histologic parameters of bone, reflecting mainly mineralization or resorption. These correlations and the finding of significant differences in plasma BGP between LT-ROD and HT-ROD indicate that plasma levels of BGP reflect bone formation in uremia and predict underlying bone histology.

Les taux plasmatiques de la Gal-protéine osseuse reflètent la formation osseuse chez les malades en dialyse chronique. La valeur des taux plasmatiques de la Gla-protéine osseuse pour la prédiction de l'histologie osseuse a été évaluée chez 30 hemodialyzés chroniques. Une biopsie osseuse a été pratiquée chez tous les malades et les prélèvements sanguins pour la mesure de la BGP, parathormone, alcaline phosphatase, calcium et phosphore ont été effectués au moment de la biopsie. L'histologie osseuse a révélé une ostéodystrophie rénale à faible niveau de remodelage (LT-ROD) chez 13 malades et une ostéodystrophie rénal à haut niveau de remodelage avec hyperparathyroidisme (HT-ROD) chez 17 malades. Les taux de BGP étaient au dessus de la normale chez les malades avec LT-ROD (47.3 ± 7.9 vs. 6.8 ± 0.2 ng/ml) et extrêmement élevés chez les malades avec HT-ROD (831 ± 170 ng/ml). Aucune différence entre les deux groupes n'a été retrouvée avec la parathormone ou alcaline phosphatase bien que BGP soit correlée avec la parathormone (r = 0.64) et la phosphatase alcaline (r = 0.85). Des corrélations significatives ont été établies entre la BGP et les paramètres cellulaires et non cellulaires de la formation osseuse (r = 0.73 à 0.91). Des corrélations plus faibles ou une absence de corrélation ont été trouvées entre BGP et les paramètres histologiques osseux de la minéralisation et de la résorption. Ces corrélations et la présence de différences significatives des taux plasmatiques de la BGP entre LT-ROD et HT-ROD indiquent que la BGP plasmatique reflètent la formation osseuse chez les malades urémiques et prédisent les anomalies histologiques osseuses.

Bone Gla-protein (BGP), also referred to as "osteocalcin," represents one of the most abundant non-collagenous bone proteins [1, 2]. It has a molecular weight of 5800 and the name reflects its content of three residues of the vitamin K-dependent amino acid, γ-carboxyglutamic acid (GLA). BGP in plasma has the same apparent molecular weight as the pure bone GLA-protein and studies indicate that the plasma protein is probably the intact bone protein [3]. With the availability of a radioimmunoassay for detection of BGP in plasma [3], it became possible to study BGP levels in various pathologic situations [4–7]. Because bone and kidney may affect blood concentrations of BGP [8, 9], patients without kidney function allowed us to study the contribution of bone to blood levels of BGP. Correlations between BGP and other serum biochemical parameters as well as histomorphometric parameters of bone in these patients might help us understand the role of BGP in bone metabolism and/or its value for predicting underlying bone histology.

In the present study we measured plasma BGP levels and other serum biochemical parameters in patients on chronic maintenance dialysis and evaluated the relationship between plasma BGP and quantitative static and dynamic parameters of bone structure, bone formation and resorption. In addition, we studied the relative values of plasma BGP, serum levels of alkaline phosphatase, and parathyroid hormone (PTH) for the prediction of histologic changes in patients with uremic osteodystrophy.

Methods

Patients. Thirty consecutive patients who were scheduled for diagnostic bone biopsies were enrolled in the study. There were 18 male patients and 12 female patients with a mean age of 42 ± 3.4 years (range, 13 to 72 years). All patients were on chronic maintenance dialysis. Twenty-six of them were on hemodialysis and four on chronic ambulatory peritoneal dialysis. Informed consent was obtained from all patients before the study. The duration of dialytic therapy was 32 ± 2.6 months (range, 2 to 108 months). All patients were anuric. None of the patients were parathyroidectomized or nephrectomized and none of them had been immobilized. No other medical problems such as liver cirrhosis, diabetes mellitus, or malabsorption were known to exist. All patients had normal outdoor activities and were on

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Table 1. Static and dynamic quantitative parameters of bone structure, bone formation, and bone resorption in 30 dialyzed uremic patients[a]

	LT-ROD	HT-ROD	Normal Controls
Parameters of bone structure			
Bone mass, %	18.1 ± 1.8[a]	27.8 ± 2.3	20.5 ± 3.9[b]
Lamellar osteoid volume, mm^3/cm^3	119 ± 21.6	20.2 ± 4.3	20.0 ± 10.4
Woven osteoid volume, mm^3/cm^3	10.1 ± 1.9	93.1 ± 14.2	0
Parameters of bone formation and resorption			
Lamellar osteoid surface, %	53.3 ± 5.0	11.7 ± 2.1	12.0 ± 4.8
Woven osteoid surface, %	4.89 ± 0.9	38.7 ± 3.4	0
Thickness of lamellar osteoid, μm	13.5 ± 2.3	10.6 ± 1.1	9.7 ± 2.8
Bone-osteoblast interface, %	3.42 ± 1.0	21.8 ± 2.5	3.4 ± 2.1
Peritrabecular fibrosis, %	1.64 ± 0.64	59.0 ± 13.4	0
Bone-osteoclast interface, %	1.69 ± 0.44	5.94 ± 0.60	1.1 ± 0.35
Parameters of bone dynamics			
Mineral apposition rate, $\mu m/day$	0.35 ± 0.06	1.07 ± 0.08	0.50 ± 0.15[c]
Labelled osteoid seams, %	7.1 ± 2.7	38 ± 6.6	64.9 ± 16.6
Mineralization lag time, *days*	78 ± 34	13 ± 1.6	20 ± 6

Abbreviations: LT-ROD, renal osteodystrophy with low bone turnover and osteomalacia; HT-ROD, renal osteodystrophy with high bone turnover and hyperparathyroid bone disease.

[a] Values are given as mean ± SE.

[b] Normal values obtained from 84 normal American subjects [12]; values are given as mean ± SD.

[c] Normal values for dynamic parameters were obtained from 28 normal American subjects.

an unrestricted diet except for potassium and fluids. The hemodialyzed patients were dialyzed three times weekly, 5 hr each, using the hollow fiber dialyzer (Cordis Dow Corp., Miami, Florida) with 1.5 m^2 surface and dialysis machines (model AK10, Travenol Laboratories, Inc., Morton Grove, Illinois). The dialysate contained 3.5 mEq calcium and 2 mEq magnesium. No concurrent therapy was given with the exception of routine dialysis support medications including folic acid, iron, vitamin B_6, phosphate binders, and multivitamins.

Bone biopsies. Biopsy specimens were taken from the anterior iliac crest using an electric drill [10]. Tetracycline hydrochloride was given (Tetracyn®, 500 mg p.o. b.i.d.) on day 19 and 20 before the biopsies. The drug was stopped for the following 12 days, and subsequently, demeclocycline hydrochloride (Declomycin® 300 mg p.o. b.i.d.) was given for 4 days. Bone biopsy specimens were obtained 4 days thereafter.

Bone histology and histomorphometry. Bone specimens were fixed in ethanol for 24 hr, dehydrated, and embedded in methylmethacrylate. Undecalcified sections of 3- and 7-μ thickness were prepared using a microtome (Model 1140, Jung, Heidelberg, Germany). The sections were stained with the modified Goldner's trichrome stain [11], which permits discrimination of calcified bone from osteoid and gives excellent cellular details [12]. Seven-micron thick unstained sections were prepared for phase-contrast and fluorescence microscopy. In addition, all slides were stained with a specific histochemical stain for detection of aluminum in bone [13]. The slides were read without knowledge of clinical or biochemical information. All sections were analyzed quantitatively for static and dynamic parameters of bone structure, bone formation, and resorption using the Osteoplan (Carl Zeiss, Thornwood, New York) according to Malluche et al [14]. A minimum of 50 optical fields were evaluated at a magnification of ×200 using an objective with 0.4 numerical aperture.

Radioimmunoassay for BGP in plasma and dialysate. At time of bone biopsies, blood samples were taken from all 30 patients for determination of plasma concentrations of BGP. BGP was measured without knowledge of bone histology in triplicate using a radioimmunoassay with rabbit antibody directed against calf BGP [3]. The antibody crossreacts with purified human BGP but not with BGP from rat or rabbit bone. Studies with peptides of known structure derived from enzymatic digestion of BGP indicate that the rabbit antibody recognizes the COOH-terminal region of the 49-residue calf bone protein. All determinations were done with four different dilutions (100, 10, 1, and 0.1 μl) to ascertain agreement between different dilutions and to assure that results were on scale. The detection limit of the assay is 0.1 ng, the intraassay variation is less than 10%, and the interassay variation is less than 15% [3].

To determine the effect of dialysis on plasma BGP levels, we measured plasma BGP in 6 of the 30 patients at the beginning and the end of a dialysis without ultrafiltration. In addition, blood samples were obtained before and after passage through the dialyzer, that is, from arterial and venous sites, and dialysate samples were taken from the outflow of the dialyzer. To determine day-to-day variations in plasma BGP levels of patients on chronic maintenance dialysis, we followed prospectively plasma BGP levels in these six patients. For this purpose, blood was drawn at least once monthly before and after dialysis for 7 to 9 months. Duration and schedule of dialysis were not changed during this time.

Biochemical measurements. Serum PTH levels were measured using a sensitive N-terminal parathyroid hormone radioimmunoassay which measures intact PTH (anti-human 1–34 PTH, courtesy of Dr. David Endres, Nichols Laboratory, San Juan Capistrano, California) [15]. Serum concentrations of calcium were measured by atomic absorption spectrophotometry (Perkin-Elmer Model 5000, Norwalk, Connecticut). Serum phosphate, alkaline phosphatase, and creatinine were measured with an autoanalyzer (model SMA 12, Technicon, Tarrytown, New York).

Statistical analysis. Linear regression analysis, multiple regression model analysis, and correlations were calculated between plasma BGP levels, PTH, alkaline phosphatase, and

Table 2. Serum biochemical parameters in 30 dialyzed uremic patients with various types of uremic bone disease[a]

	LT-ROD	HT-ROD	Normal Range
Ca, *mEq/liter*	4.7 ± 0.15	4.6 ± 0.10	4.2–5.1
P, *mg/dl*	6.14 ± 0.58	7.02 ± 0.61	2.7–4.5
Creatinine, *mg/dl*	8.80 ± 0.7	9.10 ± 0.8	0.5–1.2
AP, *IU*	71 ± 5.6	214 ± 42	20–70
iPTH, *pg/ml*	80.2 ± 9.8	195 ± 24.7	11–24
BGP, *ng/ml*	47.3 ± 7.9	831 ± 170	6.8 ± 0.2

Abbreviations: LT-ROD, Renal osteodystrophy with low bone turnover and osteomalacia; HT-ROD, Renal osteodystrophy with high bone turnover and hyperparathyroid bone disease.

[a] Values are given as mean ± SEM.

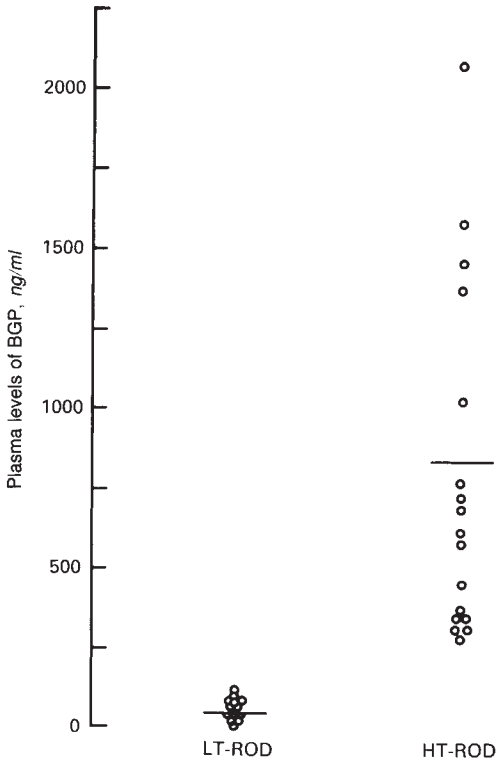

Fig. 1. *Plasma levels of bone Gla-protein in patients on chronic maintenance dialysis with low bone turnover and osteomalacia* (LT-ROD) *and with high bone turnover and hyperparathyroid bone disease* (HT-ROD).

static and dynamic parameters of bone formation and resorption. Correlations were calculated with correction for repetitive sampling.

Differences in serum biochemical parameters between the two histologic groups of uremic bone disease (see below) were calculated using the Mann-Whitney Rank Sum test for non-parametric differences. All computations were done using the Proc-Glm-In statistical analysis system #979 [16].

Results

Bone histology. Thirteen patients were found to have renal osteodystrophy with low bone turnover and osteomalacia (LT-ROD). The other 17 patients had renal osteodystrophy with high bone turnover and hyperparathyroid bone disease (HT-ROD). Histomorphometric data in these patients are shown in Table 1. Renal osteodystrophy with low bone turnover and osteomalacia was histologically characterized by accumulation of lamellar osteoid, low or normal number of osteoblasts and osteoclasts per unit trabecular surface, low fraction of actively mineralizing (doubly labelled) osteoid seams, decreased mineral apposition rate and prolonged mineralization lag time (Table 1). Stainable bone aluminum was seen in 39% of these patients. The fraction of bone osteoid interface exhibiting stainable bone aluminum ranged from 62 to 100%. The histologic features of renal osteodystrophy with high bone turnover and hyperparathyroid bone disease were high normal or elevated cancellous bone mass, accumulation of osteoid of abnormal, irregular "woven" collagen structure, abundance of osteoclasts and osteoblasts, and peritrabecular fibrosis. The fraction of actively mineralizing osteoid seams was below normal, yet higher than in patients with LT-ROD. The mineral apposition rate was high normal or elevated and mineralization lag time was normal. Stainable bone aluminum was seen in one of these patients with 12% of the osteoid-bone interface exhibiting stainable bone aluminum.

Table 3. Correlations (r-values) between histomorphometric parameters of bone and bone Gla-protein (BGP), alkaline phosphatase (AP), and parathyroid hormone (PTH)

	BGP	AP	PTH
Parameters of bone structure			
Bone mass	0.33	0.45	0.17
Lamellar osteoid volume	−0.53[a]	−0.43	−0.33
Woven osteoid volume	0.73[b]	0.69[a]	0.62[a]
Parameters of bone formation and resorption			
Lamellar osteoid surface	−0.86[b]	−0.60[a]	−0.56
Woven osteoid surface	0.84[b]	0.58[a]	0.72[b]
Bone-osteoblast interface	0.77[b]	0.66[a]	0.72[b]
Osteoblastic index	0.78[b]	0.68[a]	0.73[b]
Peritrabecular fibrosis	0.91[b]	0.76[b]	0.84[b]
Bone-osteoclast interface	0.62[a]	0.63[a]	0.56[a]
Osteoclastic index	0.69[a]	0.63[a]	0.75[b]
Parameters of bone dynamics			
Mineral apposition rate	0.91[b]	0.72[b]	0.92[b]
Labelled osteoid seams	0.48	0.54	0.79[b]
Mineralization lag time	−0.27	−0.24	−0.35

[a] $P < 0.01$.

[b] $P < 0.001$.

Serum biochemistry. There were no significant differences between serum calcium levels of patients with LT-ROD and HT-ROD (Table 2). Serum phosphate levels were elevated in both groups, but there was no difference between LT-ROD and HT-ROD. Serum alkaline phosphatase levels were at the upper normal range in patients with LT-ROD and clearly elevated in patients with HT-ROD. Serum parathyroid hormone levels were elevated in both groups of patients, and patients with HT-ROD had significantly higher circulating levels of PTH.

Plasma BGP. Plasma BGP levels were abnormal in all patients. Patients with HT-ROD had significantly higher plasma levels of BGP than patients with LT-ROD and there was no overlap in plasma BGP between the two groups (Fig. 1). The highest plasma level of BGP in LT-ROD was 121 ng/ml, and the lowest value of plasma BGP in HT-ROD was 278 ng/ml (normal,

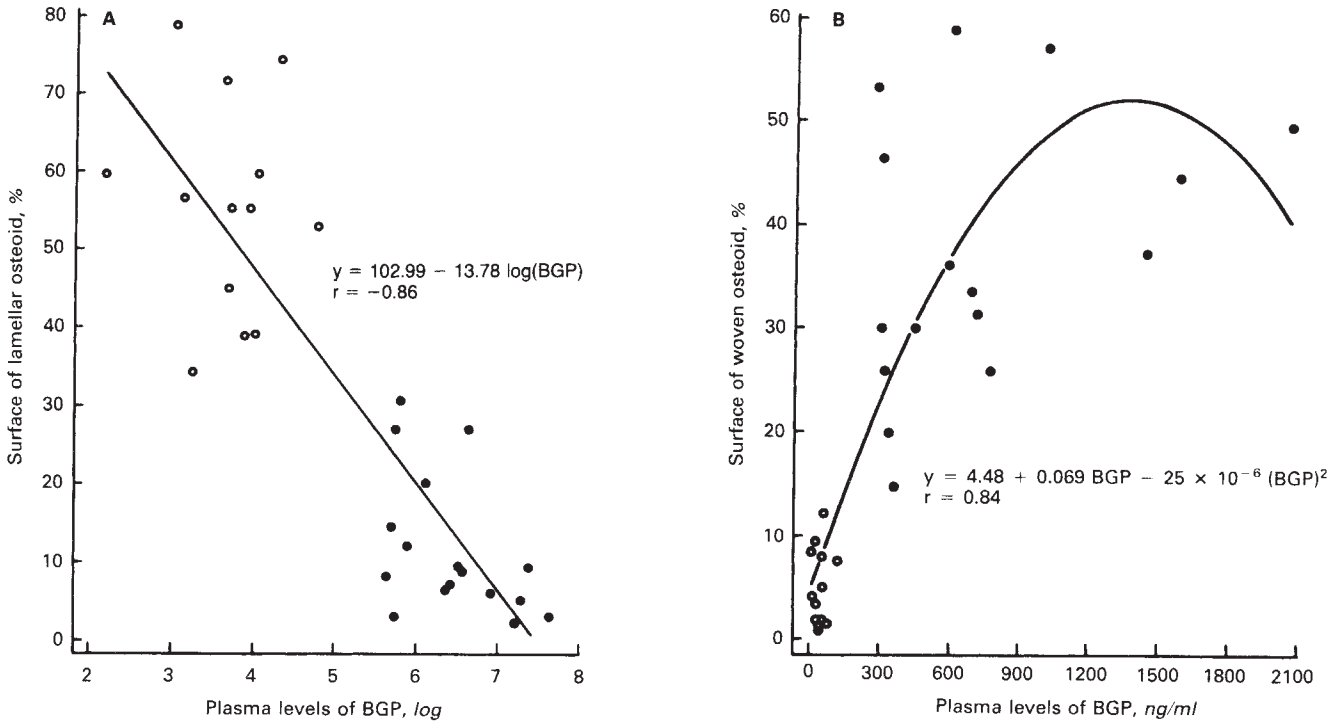

Fig. 2 A *Correlation between plasma levels of bone Gla-protein and surface of lamellar osteoid in patients with low bone turnover and osteomalacia* (○) *and with high bone turnover and hyperparathyroid bone disease* (●). **B** *Correlation between plasma levels of bone Gla-protein and surface of woven osteoid in patients with low bone turnover and osteomalacia* (○) *and with high bone turnover and hyperparathyroid bone disease* (●).

6.8 ± 0.2 ng/ml). Plasma BGP levels measured in six patients at the beginning of a dialytic therapy were not different before and after passage through the dialyzer (335 ± 81 vs. 338 ± 48 ng/ml) and no significant changes were found at the beginning and the end of a dialysis (335 ± 81 vs. 414 ± 69 ng/ml). BGP concentrations in dialysate were undetectable in five patients and 3.4 ng/ml in another patient. Plasma BGP levels drawn prospectively in six patients before and after dialysis did not change significantly during 7 to 9 months.

Correlations between plasma BGP and serum biochemistry. There were no correlations between plasma concentrations of BGP and serum levels of calcium, phosphate, and creatinine. However, plasma BGP correlated well with serum alkaline phosphatase ($r = 0.85$; $P < 0.001$) and to a lesser degree with PTH ($r = 0.64$; $P < 0.01$).

Correlations between bone histology, plasma BGP, and serum biochemistry. Cancellous bone mass correlated neither with plasma BGP, serum alkaline phosphatase, or PTH. Volume of lamellar osteoid correlated inversely with BGP but not with alkaline phosphatase or serum PTH. There was a direct correlation between volume of woven osteoid, BGP, and PTH. The surface of lamellar osteoid correlated negatively with BGP (Fig. 2A) and to a lesser degree with alkaline phosphatase and PTH. The surface of woven osteoid correlated positively with BGP (Fig. 2B) and, again, to a lesser degree with alkaline phosphatase and PTH. Other osteoblastic parameters such as bone-osteoblast interface, osteoblastic index, and peritrabecular fibrosis (Fig. 3) were also best correlated with plasma BGP and less with alkaline phosphatase and PTH. Bone-osteoclast interface and osteoclastic index correlated equally with BGP, alkaline phosphatase, and PTH. However, these correlations were weaker than those between osteoblastic parameters and BGP. Mineral apposition rate correlated well with BGP (Fig. 4) and PTH and somewhat less with alkaline phosphatase, whereas actively mineralizing osteoid seams correlated with PTH only. Mineralization lag time did not correlate with BGP, alkaline phosphatase, or PTH.

Discussion

The observed correlations between BGP and histomorphometric parameters of bone indicate that BGP is a good marker for osteoblastic activity, in particular for bone matrix and collagen fiber production. This is evidenced by a strong correlation between BGP and the volume of woven osteoid, the surface of woven osteoid, and peritrabecular fibrosis. The negative correlation between BGP and volume and surface of lamellar osteoid might reflect that, in uremic osteodystrophy, the appearance of woven osteoid is associated with less lamellar osteoid [17]. The good correlations between BGP and the number of osteoblasts, bone-osteoblast interface, and mineral apposition rate further support the notion that BGP is an indicator of osteoblastic activity. Osteoblastic activity encompasses bone formation and mineralization. The lack of correlations between BGP and parameters of bone mineralization, such as fractional labelling of osteoid and mineralization lag time, indicates that BGP reflects mainly bone formation activity by osteoblasts and not bone mineralization in our uremic patients.

It is of note that BGP levels correlate only marginally with the extent of active resorption lacunae per trabecular surface and

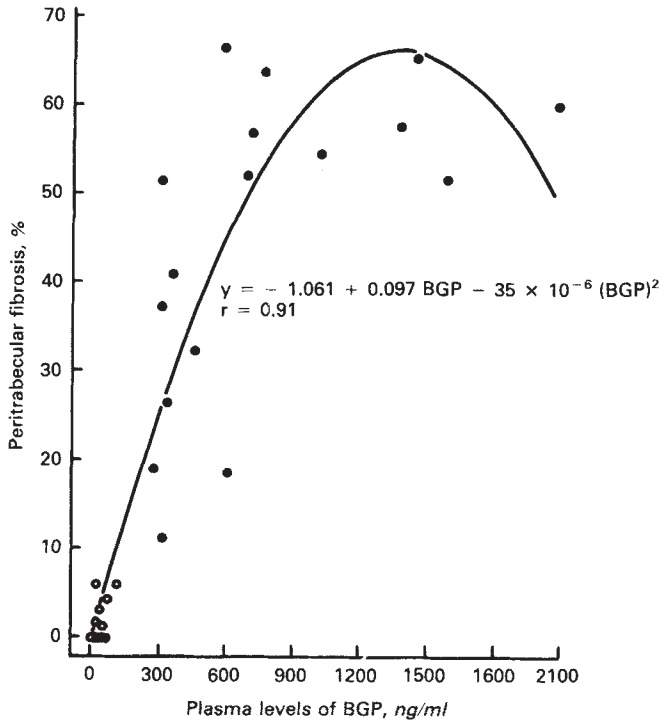

Fig. 3. *Correlation between plasma levels of bone Gla-protein and peritrabecular fibrosis in patients with low bone turnover and osteomalacia* (○) *and with high bone turnover and hyperparathyroid bone disease* (●).

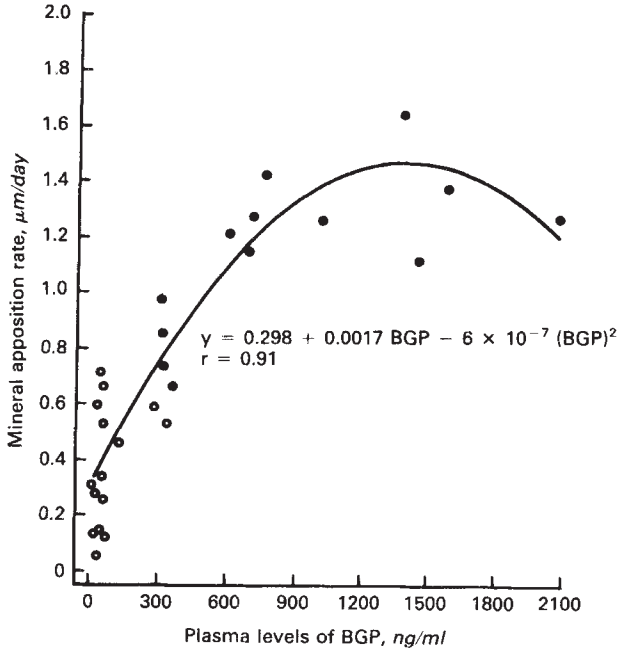

Fig. 4. *Correlation between plasma levels of bone Gla-protein and mineral apposition rate in patients with low bone turnover and osteomalacia* (○) *and with high bone turnover and hyperparathyroid bone disease* (●).

the number of osteoclasts per trabecular surface. This correlation might be spurious, reflecting the known coupling between bone formation and resorption [18].

Our results point to the osteoblasts as the most likely site of BGP production. This notion agrees with the observation that plasma BGP arises from synthesis of BGP in bone and not from release of BGP through bone resorption [8]. In addition, it is in keeping with the finding that BGP is secreted in vitro by osteosarcoma cells which have a PTH responsiveness and alkaline phosphatase activity akin to osteoblasts [19].

Our data agree with reports by Delmas et al [6] who found a correlation between plasma BGP levels and bone turnover in osteoporotic patients. However, the prevailing notion that BGP is involved in mineralization cannot be confirmed by our results. The apparent discrepancy can be explained by the fact that studies ascribing a role fo BGP in mineralization were done either in vitro [20, 21] or in growing rats [22, 23]. Our studies were done in patients with various levels of elevated BGP.

The finding that BGP correlates better with histomorphometric parameters of bone formation than alkaline phosphatase or PTH ascribes a promising role to BGP as a non-invasive tool for assessment of underlying histology. This potential is further increased by the fact that BGP, unlike alkaline phosphatase, is a bone-specific protein, that is, not produced by other organs.

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